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Metabolomics reveals altered biochemical phenotype of an invasive plant with potential to impair its biocontrol agent's establishment and effectiveness

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A plausible, but largely unexplored reason for many weed biocontrol agents failing to establish or being ineffective, could involve abiotically induced changes to an invasive plants' biochemical phenotype and consequent enhanced herbivore resistance. Considerable literature demonstrates that chemically altered plant phenotypes can impair insect life history performance. Heather beetle, (*Lochmaea suturalis*), introduced to control invasive heather (*Calluna vulgaris*) in New Zealand (NZ) was difficult to establish and displays variable effectiveness. Using UHPLC-MS non-targeted metabolomics, we analysed primary and secondary metabolites of *C. vulgaris* from its native range (Scotland) and it's introduced range (NZ), between which, differences in soil nutrients and ultraviolet light exist. We also explored secondary metabolite variation between sites within each range. New Zealand samples had the highest number of amplified metabolites, most notably defensive phenylpropanoids, supporting the concept of abiotically induced upregulation of key biosynthetic pathways. Analysis of secondary metabolite variation within each range revealed differences between sites but found little correlation of phenylpropanoid levels being influenced by variable soil nutrients. These results validate questions about the possibility of abiotically altered biochemical phenotypes in invasive plants, influencing weed biocontrol agent establishment and effectiveness, and show the potential for metabolomics in assisting future, or retrospectively analysing biological control programmes.

Globally, invasive plants are a serious threat to the terrestrial habitats they invade, causing considerable economic loss in managed production systems such as agriculture, horticulture and forestry or ecological perturbations in environmentally sensitive natural systems including communal rangelands and conservation lands which provide essential ecosystem services[1]. In most instances such intrusions cumulatively result in losses of production and biodiversity at the species, community, or ecosystem level[2].

For many of these ecosystems, the introduction of insect or pathogen biocontrol agents sourced from the plant's native range (classical biocontrol) offers a sustainable long-term management alternative for invasive plants[3,4]. However, some biocontrol programmes do not achieve the desired goals due to agents either failing to establish[3,5], being ineffective once they are established[6,7] or display variable effectiveness between sites[8]. While evidence suggests biocontrol agent population establishment is likely influenced by many environmentally driven factors[9–11], there is a paucity of literature considering target plant biochemistry and how changes in its genotype or biochemical phenotype might act as a component allee effect and impose limitations or extinctions on control agent establishment, particularly while at vulnerable low population numbers[12]. Likewise, literature addressing target plant biochemistry on agent ineffectiveness or variable inter-site effectiveness is sparse (but see[8]).

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In natural habitats, plants as sessile organisms are simultaneously subjected to multiple stressors to which appropriate responses are required for establishment, growth, and survival[13–15]. Considerable evidence relating to abiotic and biotic stresses on plants exists[16–18], demonstrating that plants are capable of responding to such stresses via a plastic and finely balanced response network involving activation of stress responsive genes which regulate phytohormone production, redox signalling pathways, growth, and calcium and protein kinase cascades, resulting in changes to both primary and secondary metabolism[17,19,20], all of which are key endogenous components in mediating plant stress responses.

Plant responses resulting from adaptive changes driven by the genotype under changed environmental conditions can manifest either independently or as a combination of morphologically, physiologically, or biochemically altered phenotypes broadly termed phenotypic plasticity[21–23]. Phenotypic plasticity is thought to be a common characteristic of invasive plants[24], but experimental evidence investigating this is equivocal[25], citing it is often limited to the early stages of establishment[26] and constrained within the ecological limits of the sites studied[14,27,28].

Nevertheless, some recent publications have demonstrated abiotically induced alterations to the metabolome of invasive plants when compared with their conspecifics in the native range. Such alterations generally result in increased levels of various defensive secondary metabolites[29–31], suggesting that this may not be an uncommon phenomenon.

Heather, (*Calluna vulgaris*) is an invasive shrub introduced from Europe now widely established on the North Islands Central Plateau (CP) of New Zealand (NZ), where it impacts this fragile sub-alpine environment[32–34]. The heather beetle (*Lochmaea suturalis*) (Coleoptera : Chrysomelidae) sourced and introduced from the United Kingdom (UK) in 1996 as a biocontrol agent, required multiple releases over several years before establishment, does not achieve the population densities of its home range in the UK and Europe and currently displays variable effectiveness within the CP region. A long-term research effort has investigated several factors to explain these scenarios with only low foliar nitrogen in CP heather providing a definitive explanation[35–38], but potential changes in plant defensive biochemistry had not been explored. We hypothesize that the plastic nature of plant biochemical responses induced potentially by abiotic factors may cause introduced biocontrol agents, to encounter plants with altered defensive biochemistry compared to those plants from where the agent was sourced. We aim therefore to explore the biochemical phenotype of this plant in the invaded range (NZ) and its native range (UK), from where the biocontrol agent was sourced and we further aimed to explore such phenotypic variability between sites within each range.

In the CP, mean seasonal temperatures for this higher altitude region, while displaying slightly greater daily variation of maximums and minimums, are very closely matched to the higher latitudes of Scotland in the UK. The CP region however experiences a longer winter period (3–4 weeks) than that of northern UK latitudes[39], but spring and early summer mean monthly temperatures, i.e., the growing period for *C. vulgaris*, are very closely matched. We assume therefore that *C. vulgaris* in this region experiences climatic conditions very similar to its northern hemisphere conspecifics. We hypothesize that two abiotic parameters of this region that may induce long term permanent changes to the biochemical profile of *C. vulgaris* are soil nutrients and light.

Soil nutrient availability (depending on the underlying geology) and long term increased chemical defences in plants (most notably phenylpropanoids such as polyphenolics, flavonoid glycosides, flavones and coumarins), are well documented[40–45]. Soils of heather dominated heathlands in the UK and Europe range from free draining to wet, are acidic and low in nutrients, especially phosphate and nitrogen[46]. Similarly, the young volcanic soils of the CP region are generally free draining but with levels even lower in phosphate and nitrogen[38,47].

Light quantity (intensity) and quality (the balance of PAR, UV-A and UV-B), which is dependent on latitude and altitude[48,49], also induce long term changes to the plant biochemical phenotype by modulating the jasmonate (JA) and abscisic acid (ABA) dependant pathways thus inducing Shikimate-phenylpropanoid derived flavonoids and phenolic acids. These metabolites play an important role in plant photoprotection but have also been shown to enhance plant defences against insect herbivores[50–53] or provide systemically acquired resistance to biotrophic pathogens[54,55]. Light intensity and ambient UV are significantly different between the two regions, with peak summertime UV index figures of 12 to 13 at the North Island CP being approximately double that of higher latitude regions of the UK at 6 to 7[48,49]. Furthermore, previous work demonstrating plasticity of secondary metabolites in *C. vulgaris* at varying altitudes[56] and seasonally[57] in Europe and in response to multiple abiotic factors in New Zealand[58,59], indicates that this plant species readily responds to changing parameters in its environment, making it perhaps an ideal model species to explore.

In recent years, high throughput mass spectral chromatographic technology in conjunction with online cheminformatic platforms has markedly progressed metabolomic analytical capability. Metabolomics makes it feasible to now study plant biochemistry at the molecular level, providing a targeted or non-targeted characterisation and quantification of currently identifiable metabolites (the metabolome), in a particular plant tissue in response to its environment or treatment[37,60–64].

In this study, we applied non-targeted metabolomics to investigate possible changes to both primary and secondary metabolites of *C. vulgaris* between its native range in Scotland (UK) and plants from the invaded range of the CP in New Zealand. Using unsupervised principal components analysis (PCA) our findings are exploratory only but do demonstrate clear differences in the plant metabolomes between the native and invaded ranges potentially linked to UV and soil nutrients.

It is beyond the scope of this study to independently assess the effects of these abiotic parameters (UV and soil nutrients) on the plant metabolome, as this requires controlled, experimental conditions. Testing the direct effects of altered foliar biochemistry on heather beetle performance is also beyond the scope of this study. These questions are now being addressed and will be presented in subsequent publications. However, we posit that our results validate the question of biochemically altered defences potentially exposing beetles at the CP sites to encounter host plants that are less assimilable than those in its native range. Furthermore, we propose that

metabolomics is a powerful analytical tool that could be useful to assess biochemical changes in invasive plants due to differing abiotic influences, and such information could assist decision making in future biocontrol programmes or retrospectively elucidate unsuccessful ones.

Materials and methods

Sampling

Foliage

Five samples of mature heather (*Calluna vulgaris*) foliage were collected from each of four sites in Scotland (S Fig. 3a), during the Northern Hemisphere summer from 29th June to 3rd July 2018. The sites were, Glensaugh (GS) Lat. 56.910605° Lon. -2.569144°, alt. 323 m, soils - Strichen, peaty gleyed podzols; Ballogie Estate (BE) Lat. 56.998852° Lon. -2.743927°, alt. 316 m, soils - Countesswells, peaty gleyed podzols; Glenturret (GT) Lat. 56.415890° Lon. -3.912876°, alt 351 m, soils – Gourdie, noncalcareous gleys with peaty gleys; Creag Meagaidh (CM) Lat. 56.933362° Lon. -4.527912°, Alt 290 m, soils – Arkaig, peaty gleys with dystrophic semi-confined peat. During this period, most plants at GS, GT, and CM, were not flowering but at BE a small number had reached very early budburst. Plants with flowers were, where possible, avoided for sampling. Each sample comprised of equal quantities of 10–15 mm long fresh sprigs combined from three individual intertwined or adjacent mature plants. Each such sample was taken ≥ than 10 m apart and all were immediately cryo-frozen in nitrogen vapour then stored at -80 °C until freeze drying and grinding.

Mature *C. vulgaris* plants were sampled from four sites from the Central Plateau region of the North Island, New Zealand (S Fig. 3b), during January 17th − 18th 2019 (Southern Hemisphere summer) using the same sampling and storage protocol. The sites were Mangaturuturu (MU) Lat. -39.303293° Lon. 175.390239°, alt. 817 m, soil - Orthic Podzol; Waiouru (WU) Lat. -39.456172° Lon. 175.677246°, alt. 814 m, soil - Orthic Allophanic; Quarry (QU) Lat. -39.431120° Lon. 175.685689°, alt. 881 m, soil - Orthic Allophanic; Waihohunu (WH) Lat. -39.227263° Lon. 175.732654°, alt. 975 m, soil - Tephric Recent. At all sites, plants were at very early to early budburst with only a few flowers present, and again plants with flowers were avoided for sampling.

Soils and UV:

Five samples for soil nutrient analysis were collected from random positions in all sites. Each sample consisted of 3 soil cores each taken to a depth of 15 cm and air dried until no change in mass. Cores were sieved through a 1 mm precision sieve, then combined and analysed for Olsen P, Total N and pH. NZ samples were analysed by Hill Labs, Hamilton NZ and SC samples at the James Hutton Institute Laboratories, Aberdeen, UK. Phosphorus for Olsen P for both NZ and SC samples were measured on air dried soil using $NaHCO_3$ (0.5 M; pH8.5) extractant. For Total N both NZ and SC samples were subjected to the Dumas combustion method then measured using a VarioMAX CN Macro Elementar analyser on NZ soils and a Thermo Flash EA 1112 elemental analyser (Thermo Fisher) on SC soils. For pH, both soils were slurried (1:2 v/v) soil:H_2O and analysed using glass bulb pH probes. Parameters used for *C. vulgaris'* exposure to ultra-violet for each range/region, are based on reviews of global summer noontime maxima of a standardised measurement of erythemal UV intensity known as the ultra-violet index (UVI) and with an adjustment for altitude (1000 masl) at the North Island Central Plateau sites[58,59,65].

Invertebrates

To assess potential herbivore induced plant metabolite responses, using a beating tray and standardised beating protocol, we collected all invertebrates from the foliage of all plants at all sites and immediately preserved them in 70% ethanol. These were later sorted into family and their associated feeding guild, enumerated, then interrogated for Pearson correlation with phenylpropanoid compound intensities associated with their own site.

Foliage sample preparation for UHPLC-MS analysis

Foliage samples were freeze dried then stored for 2 weeks at -20 °C prior to grinding to ≈ 150–50 µm particle size before extraction. 50 ± 2.0 mg of ground sample were weighed into 2 mL microcentrifuge tubes and extracted in 800 µL of pre-chilled $CHCl_3$:MeOH (1:1 v/v) with internal standards comprising 1.6 mg L-1 of d5-L-tryptophan, d4-citric acid, d10-leucine, d2-tyrosine, d35-stearic acid, d5-benzoic acid, 13C2-glucose, and d7-alanine. Samples were vortexed for 2 min and kept at -20 °C for 1 h after which 400 µL of H_2O was added and again vortexed for 2 min. Samples were then centrifuged for 15 min (11000 RPM @ 4 °C) creating a biphasic layer. Two aliquots of 200 µL each of the upper, aqueous layer were transferred to 2 ml microcentrifuge tubes and evaporated to dryness, under a continuous stream of nitrogen (30 °C for 50 min) and stored @ -80 °C until reconstitution. For semi-polar compounds, reconstitution for C18-LC-MS analysis, was in 200 µL of C_2H_3N:H_2O (1:9 v/v), vortexed for 1 min then transferred to a glass insert in an auto-sampler vial. For polar compounds reconstitution for HILIC-LC-MS analysis, was in 200 µL of C_2H_3N:H_2O (1:1 v/v), vortexed for 1 min then similarly transferred to a glass insert in an auto-sampler vial. A pooled mix of all samples was similarly prepared ($n = 7$) and used as quality controls (QC) for each of the C18 and HILIC streams. These were evenly distributed (every 8th sample) to monitor any systematic effects on the corresponding analysis. Five extraction blanks were included at the beginning of and an amino acid standard (A9906; Sigma-Aldrich, NZ) at the beginning and end of the sampling sequences.

Chromatography and mass-spectrometry

Chromatography and tandem mass-spectrometry analysis of polar and semi-polar compounds were achieved using a Thermo LC–MS system (Thermo Fisher Scientific, Waltham, MA, USA) which consisted of an Accela 1250 quaternary UHPLC pump, a PAL auto-sampler fitted with a 15,000 psi injection valve (CTC Analytics AG., Zwingen, Switzerland) and 20 µl injection loop, and an Exactive Orbitrap mass spectrometer with electrospray ionisation run in both positive and negative modes.

For semi-polar compounds, samples were cooled in the auto-sampler at 4 °C and a 2 µL aliquot was injected into a 1.9 µm Thermo Hypersil Gold C18 column (UPLC, 100 mm × 2.1 mm, Thermo Fisher Scientific, USA) at 25 °C with a gradient elution programme and a flow rate of 400 µL/min. The mobile phase was water with 0.1% formic acid (solvent A), and acetonitrile with 0.1% formic acid (solvent B). Using the Xcalibur software package provided by the manufacturer the gradient elution programme was: held at 5% B (0–0.5 min),5–99% B (0.5–13 min), held at 99% B (13–15 min), returned to 5% B (15–16 min) and allowed to equilibrate for a further 4 min prior to the next injection. The first 1.5 min and the last 6 min of the chromatogram were diverted to waste. Mass spectral data were collected in profile mode over a mass range of m/z 60–1200, at a mass resolution setting of 25,000 with a maximum trap fill time of 100 ms. Samples were run in both positive and negative ionisation modes separately. Positive ion mode parameters were: spray voltage, 3.5 kV; capillary temperature, 325 °C; capillary voltage, 50 V, tube lens 120 V. Negative ion mode parameters were: spray voltage, −3.5 kV; capillary temperature, 325 °C; capillary voltage, −90 V, tube lens −80 V. The nitrogen source gas desolvation settings were the same for both modes (arbitrary units): sheath gas, 40; auxiliary gas, 10; sweep gas, 5[66].

For polar compounds, samples were cooled in the auto-sampler at 4 °C and a 2 µL aliquot was injected into a 5 µm ZIC-pHILIC column (100 mm × 2.1 mm, Merck Darmstadt, Germany) at 25 °C with a gradient elution programme and a flow rate of 250 µL/min. The mobile phase was acetonitrile with 0.1% formic acid (solvent A) and 16 mM ammonium formate in water (solvent B). The gradient elution programme was: held at 97% A (0–1 min), 97–70% A (1–12 min), 70–10% A (12–14.5 min), held at 10% A (14.5–17 min), returned to 97% A (17–18.5 min) and allowed to equilibrate for a further 5.5 min prior to the next injection. The first 1.5 min and the last 5 min of the chromatogram were diverted to waste. Mass spectral data were collected in profile mode over a mass range of m/z 55-1100 at a mass resolution setting of 25,000 with a maximum trap fill time of 100 ms. Positive ion mode parameters were: spray voltage, 3.5 kV; capillary temperature, 325 °C; capillary voltage, 90 V, tube lens 120 V. Negative ion mode parameters were: spray voltage, −3.0 kV; capillary temperature, 325 °C; capillary voltage, −90 V, tube lens −100 V. The nitrogen source gas desolvation settings were the same for both modes (arbitrary units): sheath gas, 40; auxiliary gas, 10; sweep gas, 5[67].

Data analysis

Thermo derived .raw files for each stream i.e., C18 and HILIC in both positive and negative modes, were converted to mzML format using MSConvertGUI[68], uploaded into MZmine[69] to determine the appropriate baseline noise threshold and then into XCMS online "xcmsonline.scripps.edu", for feature detection, alignment and exploratory data analysis[70]. Feature detection parameters for C18 data were, m/z deviation 10 ppm, min and max peak width 5 and 20 respectively, mzdiff 0.001, s/n threshold 20, Prefilter intensity 1e4 and noise filter 4e4. For HILIC data the same parameters were 10ppm, 10 and 60, 0.001, 20, 1e4 and 3e4. After downloading the output, a series of procedures followed, converting raw mass spectrometry data into data matrices comprising *m*/*z*, retention time, and the corresponding ion intensity measurements suitable for statistical analysis.

Reduction of background variability in the full data matrix of each stream was performed using a QC vs. Blank t-test thus allowing subtraction of those features with $p > 0.05$ values or t.stat values corresponding to any features high in the blanks. These data matrices were each uploaded into MetaboAnalyst ver. 6.0 (MA 6.0)[71] and data integrity checked to confirm the number of samples, number of peaks, missing values, and the number of treatment groups. No missing values were detected in any of the data sets. For filtration of variables showing low repeatability, the threshold to remove those with high percent relative standard deviation (RSD) was set at 30% to that of the QCs and the data normalised by auto-scaling (mean-centred and divided by the standard deviation of each variable) and Gaussian distribution confirmed so that feature mass intensities are comparable.

For each stream, we explored the entire data matrix at the site level for both the NZ and SC ranges by subjecting them to multiple principal component analysis (PCA) in MA 6.0. This indicated a degree of site cluster overlap within each range but a clear and consistent separation of clusters between the NZ and SC ranges (see S Fig. 2). We therefore considered it appropriate to apply paired PCA to each streams data matrix at the NZ and SC level for the purpose of demonstrating those differences between the metabolomes of each range.

Using the data matrices at the NZ and SC range level, final analyses were performed using the one factor statistical platform in MA 6.0. Unsupervised paired PCA's were conducted on those $p < 0.05$ features remaining in each stream, to visualise and confirm the degree of separation between clusters (metabolomes) for each range. Then from these data matrices, features for annotation were achieved by applying a paired t-test with all features below the threshold value of FDR < 0.05[72] being retained for that purpose.

Annotations were conducted and confidence levels confirmed[73] for each metabolite by interrogating the original .raw files using Xcalibur Freestyle. Formula matches were confirmed, and mass accuracy parameters were set within +/− 10.0 ppm. For HILIC level 1 confidence, m/z and rt. results were matched against a Grasslands AgResearch in house spectral standards library (GL), based on authentic standards run under the same chromatographic conditions. For all C18 and the remaining HILIC features, level 2 and 3 (parent ion plus at least one fragment and parent ion only, respectively) confidence levels were confirmed using the MassBank.eu (https://massbank.eu/) spectral database. Where the same annotated confirmed compound (ion) appeared in both + and − modes the one with the highest intensity was included in the final data table. If a compound appeared in both C18 and HILIC streams the criteria for inclusion in the table was if they were a secondary or primary metabolite respectively.

Using the metabolites covering all three levels of confidence from Tables 1 and 2 and using the pathways analysis platform in MA 5.0 we conducted analyses to elucidate which pathways are enriched the most and provide the greatest impact on the data sets between each range.

To observe inter-site differences for secondary metabolites potentially due to soil nutrient status within each range, we ran multiple PCA on the C18 pos and C18 neg data matrices independently. Each feature in these data matrices was then subjected to one-way ANOVA in MA 6.0 to provide significant features for annotation. MA

Class/Sub Class	Name	KEGG	Stream	FC	U/D	t.stat	CL	Lib	m/z	rt.	Fragments	
Flavonoid	Apigenin 7-O-glucoside	..	C18+	3.82	↑	4.56	2		433.11	4.06	433.073	
" " " "	Kaempferol 3-O-glucoside	C12249	C18+	1.64	↑	3.29	2		449.1052	5.10	287.0532	288.0579
" " " "	Kaempferol	C05903	C18+	1.37	↑	2.55	2		287.055	5.41	119.0337	
" " " "	Dihydrokeampherol	..	C18+	2.81	↑	13.29	2		289.0713	3.58	271.0592	272.0634
Flavone a.	Luteolin	C01514	C18+	2.07	↑	4.74	3		287.0549	5.10		
Flavone b.	Tricin	C10193	C18+	2.19	↑	4.50	3		331.0829	4.06		
Flavanonol	Dihydroquercetin	C01617	C18-	1.51	↑	4.41	3		303.0524	4.77		
Flavonoid-3-O-glucuronide	Quercetin 3-O-glucuronide	..	C18-	2.46	↑	4.13	3		477.069	4.89		
Flavonoid-3-O-glycoside	Syringetin 3-O-glucoside	..	C18-	96	↑	10.28	3		507.111	4.84		
Flavan a.	8-Prenylnaringenin	C18023	C18+	1.53	↑	6.61	3		341.1401	5.03		
Flavan b.	Eriodictyol	C05631	C18+	1.83	↑	8.05	3		289.0713	4.52		
Flavonol a.	Quercetin 7-methyl ether	C10176	C18+	2.53	↑	4.44	3		317.0647	5.03		
Flavonone	Eriodictyol 7-O-glucoside	..	C18+	5.06	↑	7.23	3		451.1225	4.06		
Isoflavonoid C-glycoside	Puerarin	C10524	C18+	3.76	↑	4.31	2		417.1185	4.71	399.1123	381.1027
											297.0768	
Flavonoid	Procyanidin B2	..	C18-	1.55	↓	-3.37	2		577.1309	4.29	407.0789	
" " " "	(+)-Epicatechin	C09728	C18-	1.33	↓	-5.23	2		289.0706	4.18	245.083	205.0519
											203.072	109.0299
" " " "	Isoquercitrin	C05623	C18+	1.15	↓	-2.97	2		465.1007	4.87	305.0553	
Flavonol b.	Kaempferide	C10098	C18+	4.51	↓	-7.71	3		301.0709	5.22		
Flavonol c.	Quercetin	C00389	C18-	1.15	↓	-2.83	3		301.0355	5.91		
Flavonol d.	Myricetin	C10107	C18-	1.82	↓	-2.79	3		317.0304	5.34		
Flavonoid glycoside	Kaempferol-3-glucoside-2"-p-coumaroyl	..	C18+	1.28	↓	-2.35	3		595.145	3.53		
Chalcone	Aspalathin	..	C18-	5.36	↓	-3.05	3		451.1257	3.94		
Coumarin	Scoparone	C09311	C18+	2.61	↑	9.83	2		207.0654	5.50	121.065	
Coumarin a.	Osthole	..	HIL+	2.93	↑	4.39	3		245.114	10.83		
7-Hydroxycoumarin	6-Methoxy-7-hydroxycoumarin	..	C18+	1.44	↑	3.21	3		193.0502	4.50		
Hydroxycoumarin a.	4-Hydroxycoumarin	C20414	C18+	1.2	↑	2.62	3		163.0384	3.82		
Hydroxycoumarin b.	6,7-Dihydroxycoumarin	..	C18+	4.76	↓	-7.80	3		179.0334	4.16		
Hydroxycinnamic acid	Caffeic acid	C01481	C18+	1.22	↑	2.65	2		181.0501	3.83	163.0386	164.024
" " " " " "	O-Coumaric acid	C01772	HIL+	1.29	↑	3.06	2		165.055	8.32	121.0633	
Hydroxycinnamic acid a.	2-O-sinapoylmalate	..	C18+	5.4	↓	-9.13	3		341.0853	4.17		
Hydroxycinnamic acid b.	5-Hydroxyferulic acid	C05619	HIL-	7.27	↓	-4.97	3		209.0457	8.63		
Cinnamyl alcohol	cis-o-Hydroxycinnamic acid	..	HIL-	8.02	↓	-7.22	1	GL	163.0394	7.01	119.0487	
Polyphenolic	Chlorogenic acid	C00852	C18-	1.13	↑	2.37	2		353.0871	3.82	191.0556	192.0589
" " " "	Pantothenic Acid	C00864	C18+	1.45	↑	5.38	2		220.119	3.52	202.1077	
Diarylheptanoid	Quercetin 3-Arabinoside	..	C18+	1.35	↓	-3.41	2		435.0933	5.08	303.0483	304.0539

Table 1. Phenylpropanoid compounds annotated from all four streams. level 1 and 2 confidence compound names are in black type. Level 3 confidence names are in grey type and identified to sub class only. FC = Fold Change, U/D = up or down, CL = confidence level, Lib = GL library.

Class/Sub Class	Name	KEGG	Stream	FC	U/D	t.stat	CL	Lib	m/z	rt.	Fragments	
Benzoic acid a.	Benzoic acid	C00180	C18+	1.46	↓	-4.96	3		123.0441	4.20		
Hydroxybenzoic acid	5-Hydroxysalicylic acid	..	C18+	1.77	↓	-4.23	3		155.0338	4.30		
Phenylacetic acid	Homogentisic acid	C00544	HIL+	2.44	↓	-4.19	3		169.0498	8.97		
Aminobenzoic acid	4-Aminobenzoic acid	C00568	HIL+	1.47	↑	3.56	3		138.0551	9.81		
Phenol	Phenol	C00146	HIL+	1.41	↑	2.36	3		95.0496	12.25		
Benzenediol	Pyrocatechol	C00090	HIL-	3.28	↓	-6.21	2		109.0285	8.88	108.0217	
Fatty acid	3-Hydroxy-3-methylglutaric acid	..	C18-	4.88	↓	-7.30	2		161.0443	1.54	101.0238	
Sesquiterpenoid	Isorhamnetin 3-O-glucoside	..	C18+	2.67	↑	5.04	2		479.1189	5.03	318.07	317.0655
Sesquiterpenoid a	Syringaldehyde	..	HIL-	1.78	↓	-3.86	3		181.0499	2.36		
Alpha-Amino Acid	L-Phenylalanine	C00079	HIL+	2.62	↑	9.64	1	GL	166.0867	9.79		
" " " " " "	L-Tyrosine	C00082	HIL+	1.71	↑	3.39	1	GL	182.0812	11.53	136.0762	
" " " " " "	Alanine	C01401	HIL+	1.32	↑	4.21	1	GL	90.0553	12.58		
" " " " " "	L-Threonine	C00188	HIL+	1.42	↑	3.87	1	GL	120.0659	12.77	102.0565	
" " " " " "	L-Serine	C00716	HIL+	1.56	↑	4.49	1	GL	106.0502	13.68		
" " " " " "	L-Glutamine	C00064	HIL-	2.38	↑	7.26	1	GL	145.061	13.21		
" " " " " "	Citrulline	C00327	HIL+	2.46	↑	7.48	2		176.1028	13.80	159.078	113.071
Alpha-Amino Acid a.	1-Aminocyclopropane-1-carboxylic acid	C01234	HIL+	1.57	↑	3.93	3		102.0555	12.77		
Amino Acid	L-Glutamate	C00025	HIL-	1.29	↑	3.18	1	GL	146.0451	13.12		
" " " "	L-Aspartic acid	C00049	HIL+	1.58	↑	4.39	1	GL	134.0449	14.07	116.0336	88.0387
Amino Acid a.	Betaine	C00183	HIL+	2.53	↑	6.76	3		118.0868	9.21		
Amino Acid b.	L-Pyroglutamic acid	..	HIL+	1.75	↑	6.35	3		130.0502	13.19		
Non-Protein Amino Acid	gamma-Aminobutyric acid (GABA)	C00334	HIL+	1.5	↑	3.76	1	GL	104.0708	12.01		
Amino acid conjugate	N-Fructosyl tyrosine	..	HIL+	1.38	↑	3.36	2		344.1349	8.33	187.1336	
Alpha-Amino Acid	L-Asparagine	C16438	HIL+	1.42	↓	-2.56	2		133.0614	13.58	87.0554	
" " " " " "	L-Tryptophan	C00078	HIL+	1.18	↓	-1.45	1	GL	205.0975	10.45	188.0725	
Tricarboxylic acid	cis-Aconitic acid	C00417	HIL-	1.84	↑	7.60	2		173.0086	9.46	129.0178	
Glycerolipid	Naringenin 7-O-glucoside	..	C18-	2.75	↑	4.02	2		433.1124	4.69	313.0657	
Alpha Hydroxy acid	2-Hydroxyisobutyric acid	..	HIL+	1.47	↓	-3.46	3		105.0552	9.16		
lactone	Gamma-Decalactone	..	C18+	1.54	↓	-3.00	2		171.1382	5.05	153.1275	135.1168
											154.1309	136.1202
Carbohydrate	Galactaric acid	C00879	HIL-	1.56	↑	4.14	3		209.0298	14.54		
Carbonyl compound	2-Hexenal	..	HIL+	1.38	↓	-2.33	3		99.0807	9.04		

Table 2. Primary and secondary compounds annotated from all four streams. level 1 and 2 confidence compound names are in black type Level 3 confidence names are in grey type and identified to sub class. FC = Fold Change, U/D = up or down, CL = confidence level, Lib = GL library.

6.0 also provides PERMANOVA analysis results to assess the significance of difference between PCA derived clusters. Annotated compounds were subjected to Minitab v 21.1.0 for Tukey post hoc allocation of significant differences. Phenylpropanoid compounds resulting from these analyses were graphed then individually subjected to Pearson correlation analysis to explore potential relationships with site soil nutrient status. The soil nutrient samples being randomly located within each site are therefore not paired with each foliage sample, so the mean site values for Olsen P and Total N were used.

Soil analysis results from the NZ range for Olsen P were converted from volumetric mg lt^{-1} to gravimetric mg kg^{-1} [74] to match the SC result output and together with Total N and pH were for inter-site statistics subjected to one-way ANOVA with Tukey post hoc allocation analyses. Between NZ and SC range statistics for the same parameters were achieved using t-tests. Both analyses were achieved using Minitab v 21.1.0.

Results

Paired PCA plots for both C18 and HILIC streams in both ionisation modes display clear unsupervised clustering of all samples from New Zealand's Nth Is. Central Plateau (CP) and those of Scotland (SC) in the United Kingdom (UK), see Fig. 1. Separation between the NZ and SC clusters for C18 pos is explained by principal components 1 and 2 combined being 34.9% of the observed variance. These values are for C18 neg, 36.3%, HILpos, 44.6% and HILneg, 41.5% respectively. The MA 6.0 platform provides statistical testing to verify robustness of the clustering using PERMANOVA with 999 permutations. With (PERMANOVA); C18 pos, $F=27.17$, $p=0.001$; C18 neg $F=14.77$, $p=0.001$; HILIC pos $F=38.95$, $p=0.001$ and HILIC neg $F=38.73$, $p=0.001$ respectively, these results confirm the validity of the PCA analyses.

Interrogation of the .raw files resulted in 66 metabolites being annotated from the C18pos, C18neg, HILICpos and HILICneg streams combined (See S Fig. 1a and b). Twenty-two flavonoids, five coumarins, five hydroxycinnamic acids, two polyphenolics and one diarylheptanoid all phenylpropanoids, were revealed from both C18 pos and neg streams combined. Fourteen of these were confirmed at level 2 with the remainder being level 3 (Table 1). Of these 35 phenylpropanoids, 22 of them are amplified in the NZ samples compared to those of SC. Additionally, secondary metabolites including 4 benzoic acids, 2 phenols, 1 fatty acid plus 2 sesquiterpenoids, were identified from both streams (Table 2). For primary metabolites HILIC separation revealed 16 amino acids for which the Grasslands in house spectral standards library (GL) confirmed ten to be of confidence level 1, six to confidence level 2 and the remainder to level 3. Of these 16 amino acids, 14 are

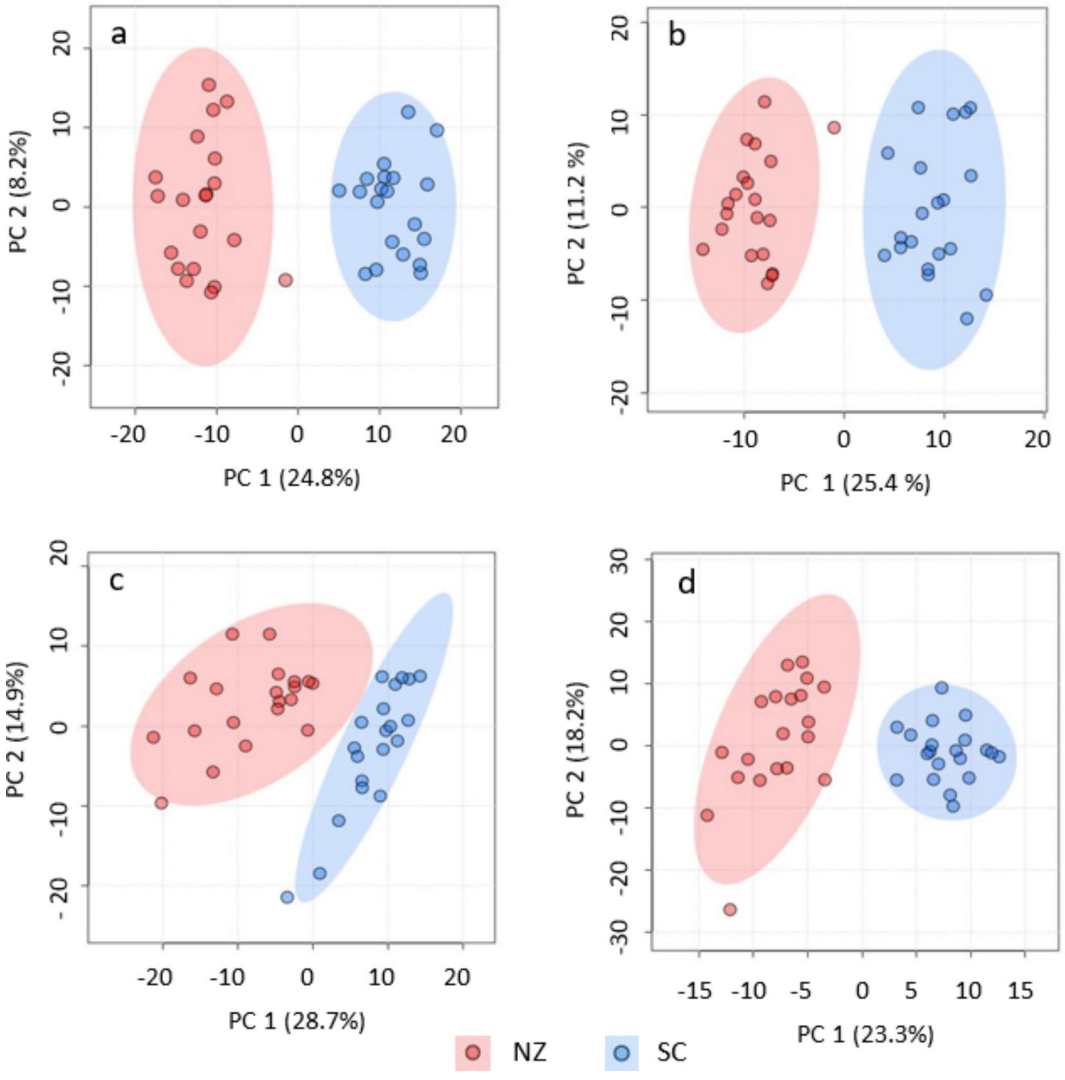

Fig. 1. Paired PCA plots including 95% confidence ellipses for each stream showing significant separation between New Zealand (NZ) and Scotland (SC) sites for each ionization mode. PC 1 and PC 2 scores for each, explain the observed variance between clusters. a = C18 pos, b = C18 neg, c = HILIC pos and d = HILIC neg. PERMANOVA (999 permutations) provides verification of the robustness of the cluster formations in all four analyses. C18 pos, $F=27.17$, $p=0.001$; C18 neg $F=14.77$, $p=0.001$; HILIC pos $F=38.95$, $p=0.001$ and HILIC neg $F=38.73$, $p=0.001$.ara>

amplified in the NZ samples. Carboxylic acids, glycerolipids and organooxygen metabolites from both streams make up the remainder (Table 2).

Pathway analysis identified those metabolic pathways to be enriched as indicated by the impact factor (x axis) and its *p* value of significance (y axis). Pathways with an impact factor ≥ 0.1 are labelled. The most significant of these were Alanine, aspartate and glutamate metabolism which revealed the greatest enrichment value of 0.77 followed by flavone and flavonol biosynthesis 0.7 and Phenylalanine metabolism 0.47 Note: The Isoquinoline alkaloid biosynthesis pathway is dependent on one compound only i.e., L-Tyrosine (Fig. 2).

Multiple PCA using C18 pos and neg matrices revealed some overlap of clusters between sites in both ranges (Fig. 3). For the NZ sites, in C18 pos MU and WH are clearly separated but display a degree of overlap for QU and WU with a combined value for PC 1 and PC 2 of 29% explaining the observed variance between these groups. Separation is less well defined except for site WH in the C18 neg matrix however with a PC combined value of 30.3%. The SC sites generally display more overlap but BE displays the greatest separation in the C18 pos stream with a PC value of 28.7%. Separation of the SC C18 neg are the least well defined with a PC value of 30.9%. Statistical testing using PERMANOVA (999 permutations) however provides verification of the robustness of the cluster formations in all four of these PCA analyses. (PERMANOVA); NZ C18 pos, $F = 28.23$, $p = 0.001$; NZ C18 neg $F = 15.87$, $p = 0.001$; SC C18 pos $F = 17.86$, $p = 0.001$ and SC C18 neg $F = 9.48$, $p = 0.001$.

Of the one-way ANOVA identified features that differ significantly (all with $p < 0.01$) between the four sites in both NZ and SC, thirty-one compounds were annotated. Seventeen of these (see S Fig. 1c) are additional to those listed in Tables 1 and 2. The previously annotated compounds are marked with and asterisk, see Table 3. Of these additional seventeen, eleven are phenylpropanoids with the remainder comprising five benzenoids and one organooxygen compound.

Ten phenylpropanoids for the NZ sites and eleven for the SC sites are graphically displayed in Fig. 4a and b respectively and were explored for Pearson correlation coefficient with site mean Olsen P and Total N values. There was little evidence either positive or negative for correlation between most of these phenylpropanoids. A moderate to weak negative but significant correlation does exist for the NZ compounds of trans-Ferulic acid (Olsen P, $r(18) = -0.43$, $p < 0.05$ and Total N, $r(18) = -0.48$, $p < 0.05$) and Epigallocatechin (Olsen P, $r(18) = -0.43$, $p < 0.05$ and Total N, $r(18) = -0.58$, $p < 0.01$). In the SC compounds slightly stronger moderate

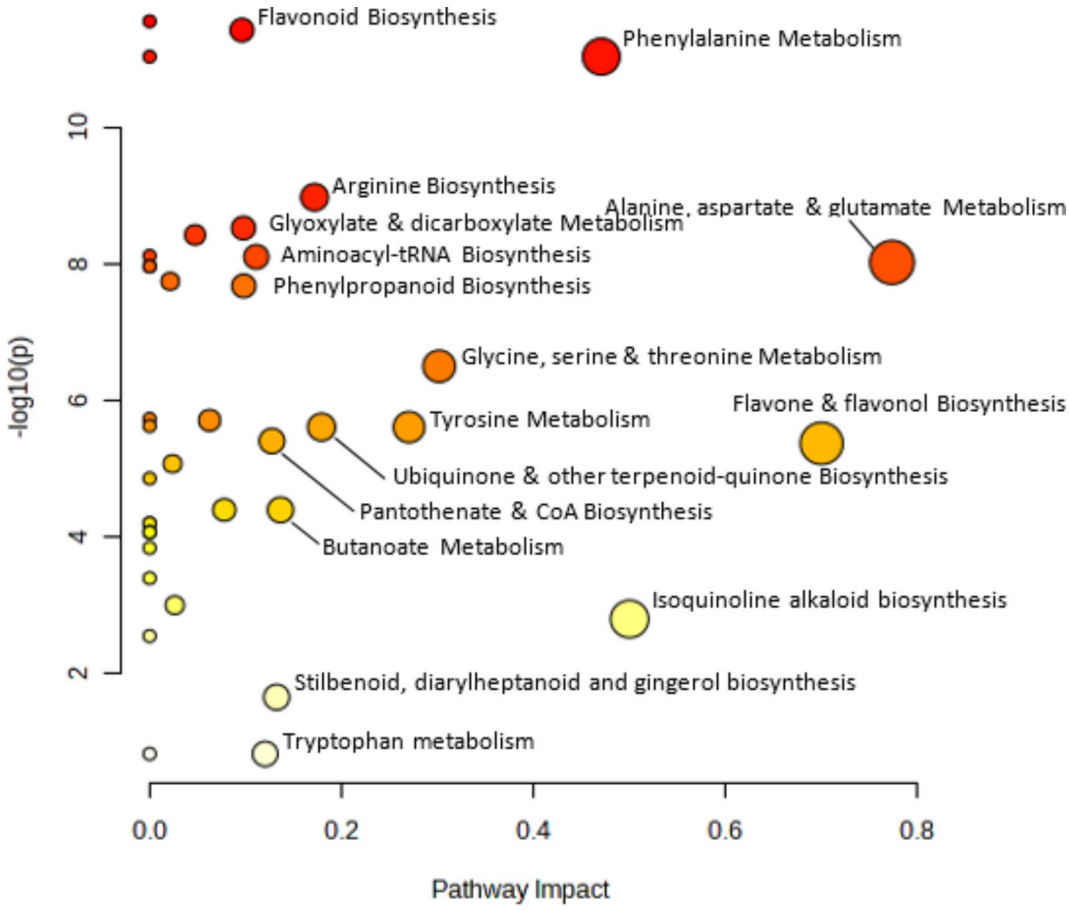

Fig. 2. Pathway enrichment analyses indicating the pathways having the greatest impact factor (x axis) and greatest *p* value of significance (y axis) between the two treatments (Ranges). Circle size indicates pathway impact value, and colour the *p* value. All pathways with an impact factor ≥ 0.1 are identified. Those in white to pale yellow are represented by one compound only.

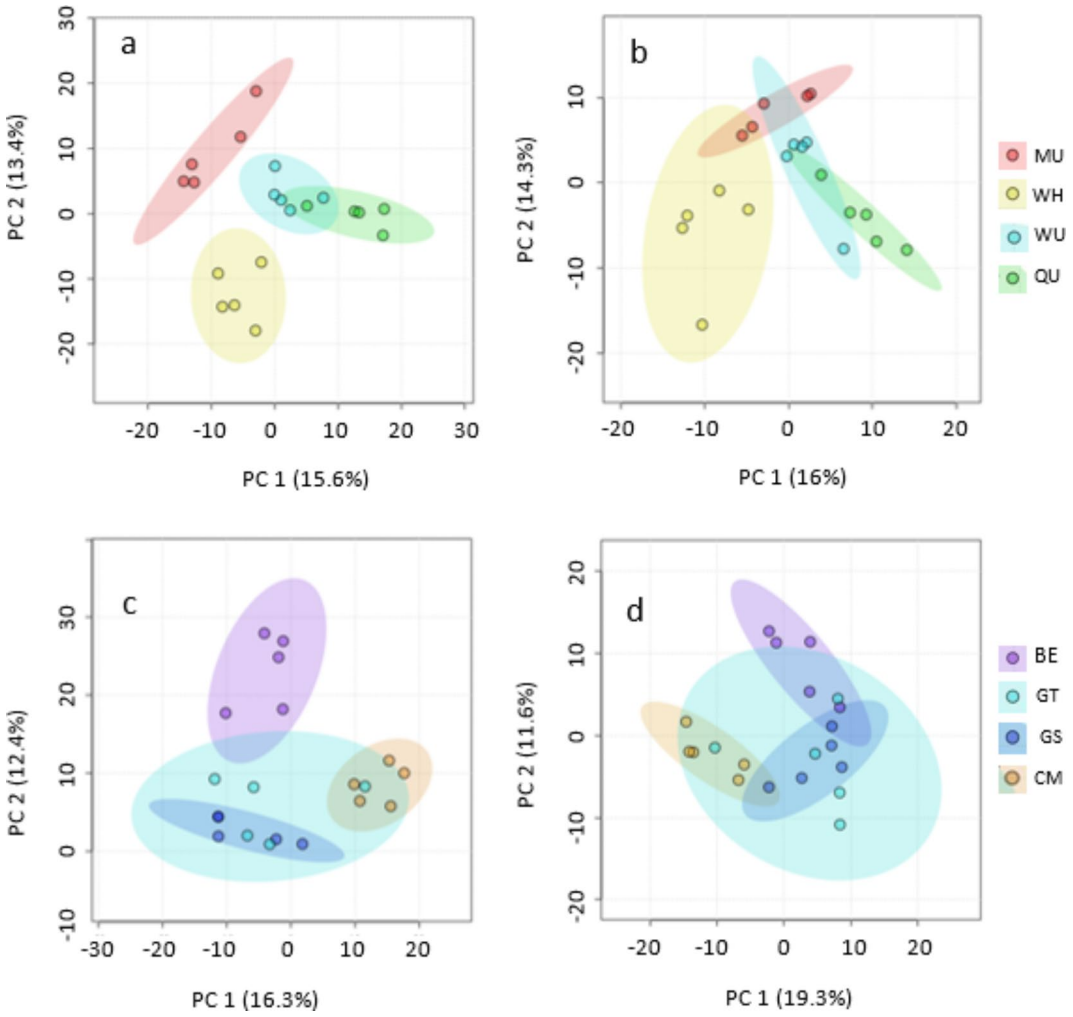

Fig. 3. Multiple PCA's of NZ and SC C18 pos and neg matrices showing clustering of sites in both ranges with 95% confidence ellipses. PC 1 and PC 2 scores for each, explain the observed variance between clusters. For NZ C18 pos (a) MU and WH clearly separate but with a degree of overlap for QU and WU. NZ C18 neg (b), separation is less well defined except for WH. SC C18 pos reveals BE, with the greatest separation but considerable overlap with the remaining three clusters. SC C18 neg separation is the least well defined. PERMANOVA (999 permutations) provides verification of the robustness of the cluster formations. For NZ C18 pos, $F = 28.23$, $p = 0.001$; NZ C18 neg $F = 15.87$, $p = 0.001$; SC C18 pos $F = 17.86$, $p = 0.001$ and SC C18 neg $F = 9.48$, $p = 0.001$. New Zealand CP sites are Mangaturuturu (MU), Waihohonu (WH), Waiouru (WU) and Quarry (QU). Scotland sites are, Ballogie Estate (BE), Glenturret (GT), Glensaugh (GS) and Creag Meagaidh (CM).

negative correlation with Total N only, exist for 2-O-Sinapoylmalate (Total N, $r(18) = -0.58$, $p < 0.01$), 6,7-Dihydroxycoumarin (Total N, $r(18) = -0.61$, $p < 0.01$) and a positive correlation for 4-Methoxycinnamic acid (Total N, $r(18) = 0.59$, $p < 0.01$).

Phytophagous invertebrates were absent from the samples collected from the New Zealand CP sites. This was because the heather beetle biocontrol agent was not established at these sites and the only known New Zealand native spp. known to graze on *C. vulgaris* are the manuka beetle (*Pyronota festiva*)[34] and an unidentified lepidopteran leaf tying caterpillar. These two native spp. are generally only present in very low numbers earlier in the season. From the SC sites, foliage dwelling invertebrates sorted into taxonomic groups and feeding guild resulted in three major taxa being identified. These were, piercing/sucking hemiptera, leaf chewing coleoptera and leaf chewing lepidoptera, (See S Table 1). Hemiptera were significantly different between sites with one-way ANOVA indicating GT significantly different from CM and BE but not GS, $F(3, 16) = 4.58$, p 0.017. Coleoptera with numbers approximately an order of magnitude higher at site GT differed significantly from CM, BE and GS, $F(3, 16) = 10.72$, p 0.001 and lepidoptera showed no significant difference between any site $F(3, 16) = 3.04$, p 0.115.

Soil nutrient analyses confirm soils of the 4 sites in SC to be relatively acidic and with very low levels of Olsen P and Total N. The volcanic soils of the CP in NZ however, while less acidic are even poorer in nutrient status with both Olsen P and Total N at extremely low levels, indeed Olsen P is at the limit of detection for

Class/Sub class	Name	Range	KEGG	Stream	CL	m/z	rt.	Fragments
Phenylpropanoids								
Flavonoid	Quercitrin	NZ	C01750	C18+	2	449.1084	4.09	287.057
" " "	Isoquercitrin *	NZ	C05623	C18+	2	465.1007	4.86	305.0553
" " "	Epigallocatechin	NZ	C12136	C18-	2	305.0651	4.00	221.0453 219.0654 261.0716 275.0559
Flavonol c.	Quercetin *	NZ	C00389	C18-	3	301.0355	5.91	
Cinnamic acid	trans-Cinnamic acid	NZ	C00423	C18+	2	149.0596	3.94	132.052 132.0593 131.416
Hydroxycinnamic acid	trans-Ferulic acid	NZ	C01494	C18+	2	195.0645	4.35	177.0529
Coumarin	7-methoxy-4-methylcoumarin	NZ	..	C18+	2	191.0705	3.94	119.0822 120.0617 120.0525
" " "	6-Methylcoumarin	NZ	..	C18+	2	161.0598	3.94	162.063 117.0698
Diarylheptanoid	Quercetin 3-Arabinoside *	NZ	..	C18+	2	435.0933	5.08	303.0483 304.0539
Chalcone	Phloretin	NZ	..	C18+	2	275.0906	5.40	107.0494
Benzenoids								
Methoxyphenol	4-methoxyphenol	NZ	..	C18+	2	125.06	3.94	110.063
" " " "	Eugenol	NZ	C10453	C18+	2	165.0908	4.99	135.0803
" " " "	Homovanillic acid	NZ	C05582	C18+	2	183.065	5.02	72.0807
" " " "	4-Hydroxybenzoic acid	NZ	C00156	C18-	2	137.0239	3.16	93.0341
Organooxygen Comp	Pantothenic Acid *	NZ	C00864	C18+	2	220.119	3.52	202.1077
Phenylpropanoids								
Flavonoid	(+)-Epicatechin *	SC	C09728	C18-	2	289.0706	4.18	245.083 205.0519 203.072 109.0299
" " "	Naringenin	SC	..	C18+	2	273.0761	4.20	123.0426
" " "	Kaempferol 3-O-glucoside *	SC	C12249	C18+	2	449.1052	5.10	287.0532 288.0579
" " "	Procyanidin B1	SC	..	C18-	2	577.131	4.57	425.0901 287.0585 125.025
Flavonol b.	Kaempferide *	SC	C10098	C18+	3	301.0709	5.22	
Flavonol c.	Quercetin *	SC	C00389	C18-	3	301.0355	5.91	
Hydroxycinnamic acid	p-Coumaric acid	SC	C00811	C18+	2	165.0546	3.94	127.9857 126.0209 119.0481
Hydroxycinnamic acid a.	2-O-sinapoylmalate *	SC	..	C18+	3	341.0853	4.17	
Cinnamic acid a.	4-Methoxycinnamic acid	SC	..	C18+	3	179.0702	4.18	
Hydroxycoumarin b.	6,7-Dihydroxycoumarin *	SC	..	C18+	3	179.0334	4.16	
Hydroxycoumarin c.	6-Methoxy-7-hydroxycoumarin *	SC	..	C18-	3	191.0349	4.63	
Benzenoids								
Benzenoid	Benzoic acid *	SC	C00180	C18+	3	123.0441	4.19	
" " " "	Gallic acid hexoside	SC	..	C18-	3	331.0671	3.30	
Benzoic acid	Homogentisic acid *	SC	C00544	C18-	3	167.0342	4.63	
Organooxygen Comp	Feruloyl quinic acid	SC	..	C18+	2	369.1191	4.60	177.0528 178.0604
Lactone	Gamma-Decalactone *	SC	..	C18+	2	171.1382	5.05	153.1275 135.1168 154.1309 136.1202

Table 3. Compounds annotated from the C18 pos and C18 neg streams within the New Zealand (NZ) and Scotland (SC) ranges. Level 2 confidence compound names are in black type . Level 3 confidence names are in grey type and identified to sub class only. CL = confidence level, Compounds in common with table 1a and 1b denoted by *.

this analytical method[74]. The paired t-test values between NZ and SC are highly significant for Olsen P $t(1, 19)$ 5.45, $p < 0.0001$; Total N $t(1, 19)$ 4.73, $p < 0.0001$, and pH $t(1, 19)$ -18.4, $p < 0.0001$ respectively. One way ANOVA for Olsen P levels at the NZ sites show no significant difference $F(3, 16) = 2.06$, $p = 0.1446$ while for Total N $F(3, 16) = 6.39$, $p = 0.0046$ and pH $F(3, 16) = 4.70$, $p = 0.015$, do show a significant difference between these means. For the SC sites Olsen P $F(3, 16) = 6.55$, $p = 0.0042$, Total N $F(3, 16) = 12.97$, $p = < 0.0001$ and pH $F(3, 16) = 16.01$, $p = < 0.0001$, all show a significant difference between the means, Table 4.

Discussion

Our results show significant differences in the biochemical profile of *Calluna vulgaris* plants growing in its native range in the UK compared to its conspecifics in NZ. A notable feature of this difference is the greater number of phenylpropanoids and amino acid metabolites that are amplified in the NZ range (Tables 1 and 2). According to the literature, both soil nutrients and ultraviolet light influence and upregulate the same major shikimate-phenylpropanoid pathway[75,76] and the subsequently dependent flavonoid and flavone/flavonol biosynthesis pathways. We provide good evidence for upregulation of all these pathways, and the resultant amplification of many primary and secondary metabolites which suggests that *C. vulgaris* is responding to differing environmental factors in these two ranges.

New Zealand CP sites have poorer soil nutrient status compared to the SC sites. Literature demonstrating depleted or low soil nitrogen and phosphate availability, increasing the level of phenylpropanoid chemical defences in plants, is provided by several studies[40,41,43–45]. Nitrogen deficiency can induce the accumulation of foliar phenylpropanoid compounds by increasing the activity of phenylalanine ammonia-lyase (PAL), a key enzyme of the shikimic acid – phenylpropanoid pathway[41,44], resulting in increased levels of total phenolics[77,78] as well as coumarins, anthocyanins, flavonoid glycosides, flavones, iso-flavones, and tannins[41,45]. Similarly, low levels of phosphate can increase foliar levels of caffeoylquinic acids, coumarins, anthocyanins and flavonoid glycosides[41,44]. Many of these compounds appear to be amplified in our NZ samples, supporting the existing evidence of low soil nutrients enhancing chemical defences.

In contrast to soils, NZ has considerably higher ambient ultra-violet intensity levels than in the UK[48,49]. Changes in the light environment, particularly increased levels of UV-B, induce signal transduction pathways to regulate plant physiological activity[40,79] often resulting in soluble phenolic compounds, particularly phenolic acids, flavonoids, flavonol glycosides of kaempferol, quercetin and myricetin as well as hydroxy cinnamic esters to accumulate in plants, many of which play an important role in photoprotection and defence[80,81].

Considerable literature exists demonstrating that such UV-radiation induced phenylpropanoids can not only increase plant resistance to insects and impair insect herbivore performance[42,50,52] but also increase plant resistance to biotrophic pathogens[54,55]. With reference to insect herbivores, several flavonoids[82,] as well as chlorogenic acid[83], several catechins[84], caffeic and o-coumaric acid[85] have all been demonstrated to impair growth and survival rates, fertility, fecundity and population growth rates in a range of insect families and we found amplified levels of many of these compounds at our NZ sites.

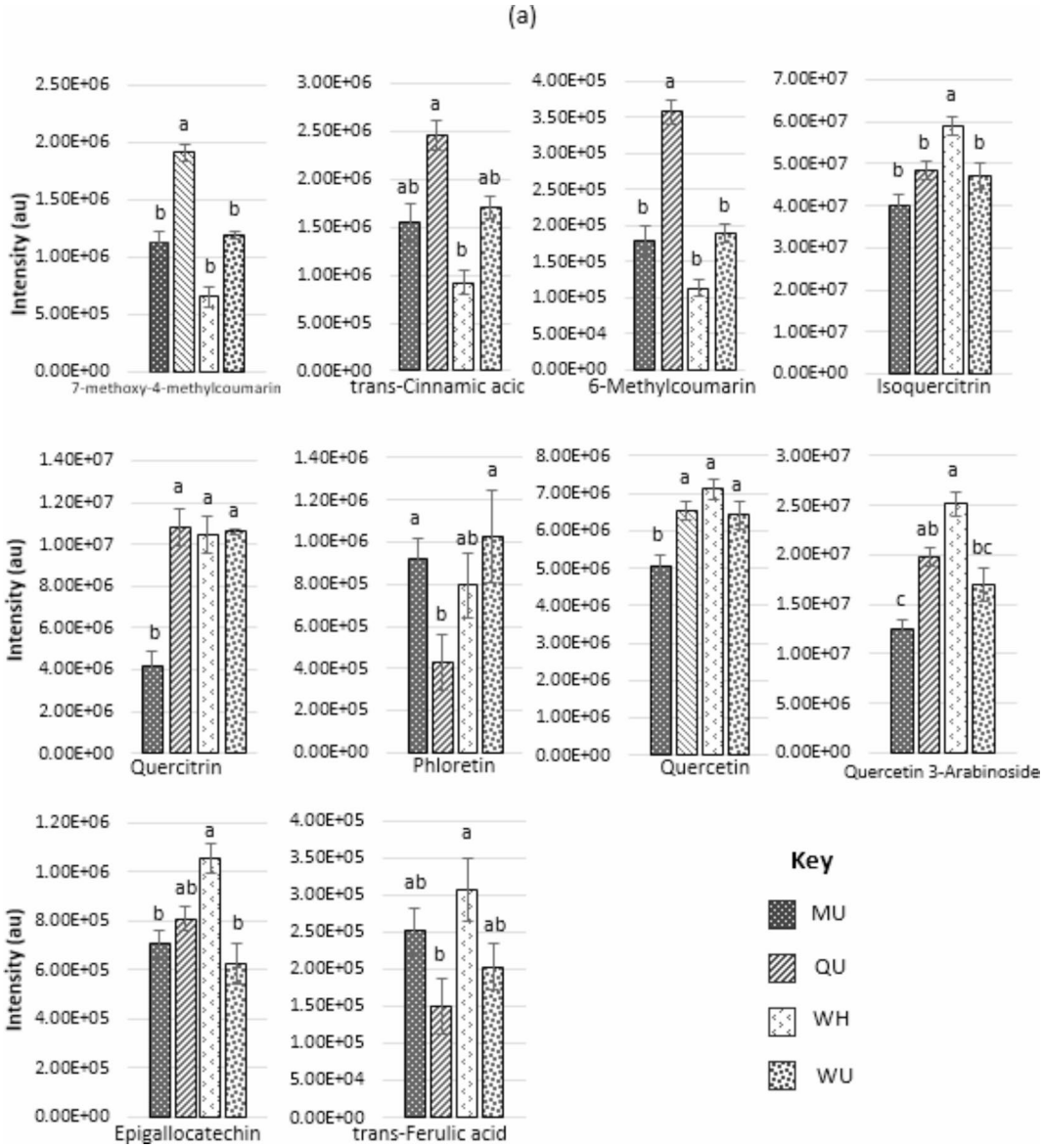

Fig. 4. (**a**) One-way ANOVA determined 10 phenylpropanoid compounds that differed significantly between the four New Zealand (CP) sites. Those sites sharing letters are not significant. All are $p < 0.01$. Sites are Mangatururu (MU), Quarry (QU), Waihohonu (WH) and Waiouru (WU). (**b**) One-way ANOVA determined 11 phenylpropanoid compounds that differed significantly between the four United Kingdom (SC) sites. Those sites sharing letters are not significant, all are $p < 0.01$. Sites are Ballogie Estate (BE), Creag Meagaidh (CM), Glensaugh (GS) and Glenturret (GT).

Of the primary metabolites, the non-protein amino acid γ-Aminobutyric acid (GABA) is also upregulated in our samples. This compound accumulates under a range of abiotic stresses including soil nutrients and light and is known to function directly in plant immune responses to biotrophic pathogens and fungi as well as being a powerful neuromuscular and growth inhibitor against insect herbivores, often causing ill thrift and death[86,87]. It's therefore conceivable, that elevated levels of some of the metabolites we have recorded in *C. vulgaris* in this study could render the assimilability of the host plant more challenging to *L. suturalis* in its new environment. These amplified metabolites may contribute to a nutritional cascade, exacerbating the effects of low foliar N levels reported in another recent paper[38] and may help explain the difficulties with the initial establishment of this control agent released to control *C. vulgaris*. in NZ.

When investigating differences between sites within the same range, we assume no variation in UV intensity exists and we know there is no influence of shade or aspect at either one, given all sites are in open shrubland communities. Therefore, we focused our analysis on response variables potentially linked to soil nutrients. While PCA revealed some overlap between sites (clusters) within each of the ranges, the one-way ANOVA revealed significant differences in phenylpropanoid compounds between some of those sites. For the CP sites we encountered significant differences in the intensities of ten phenylpropanoids with only trans-Ferulic acid and

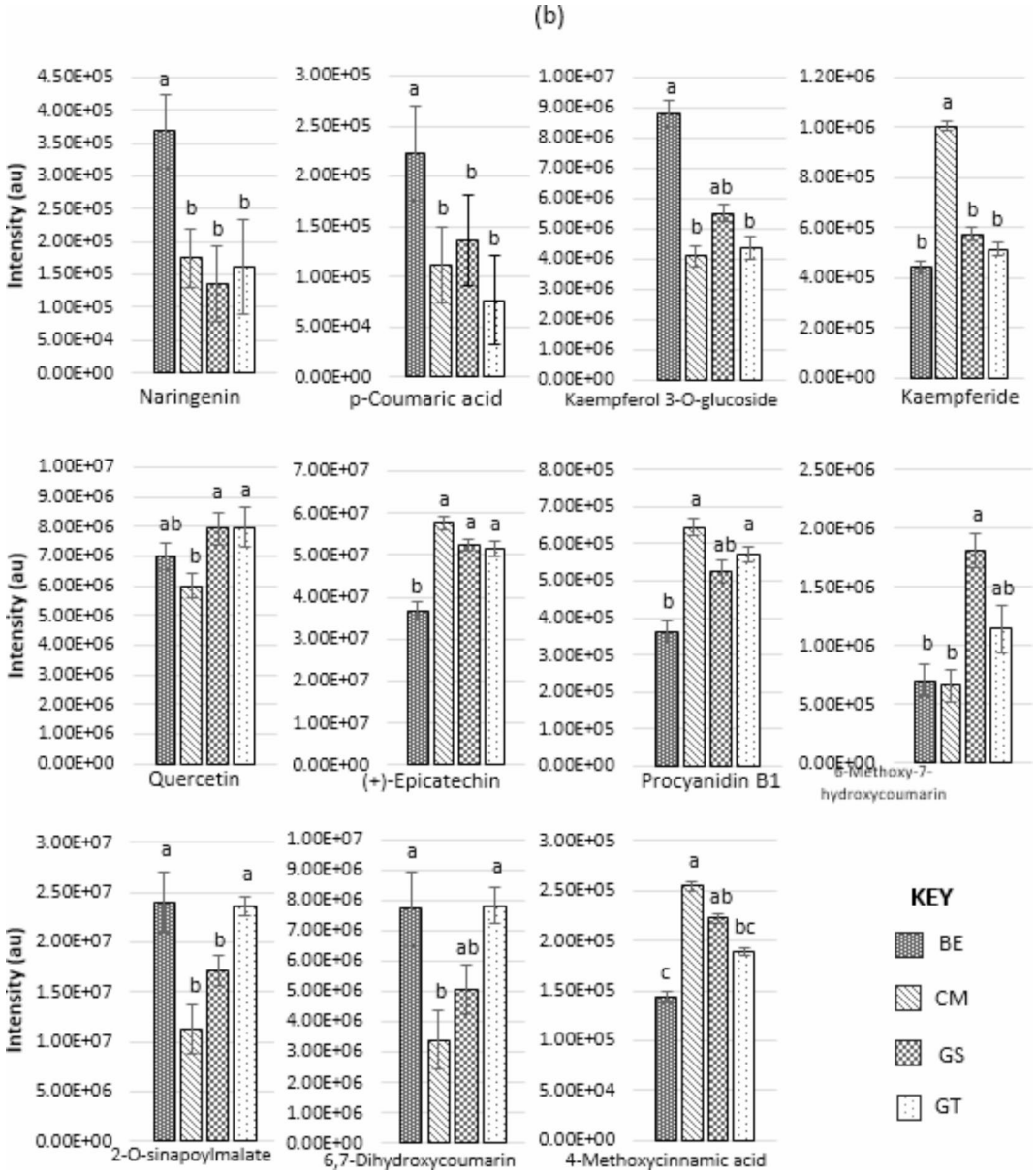

Figure 4. (continued)

epigallocatechin however, having a negative correlation with both phosphate (Olsen P) and nitrogen (Total N) levels in those soils. Overall, there remains only weak evidence that soil nutrients in this region are influencing the intensities of the phenylpropanoids that we were able to positively annotate.

The SC sites revealed eleven phenylpropanoid compounds showing significant intensity differences between sites but similarly provide little evidence (notwithstanding the results for 2-O-Sinapoylmalate, 6,7-Dihydroxycoumarin and 4-Methoxycinnamic acid) from our Pearson correlation coefficients, that soil phosphate and nitrogen levels are greatly influencing phenylpropanoid intensities. We cannot therefore, claim a causal effect as other factors and their interaction may also contribute to the observed metabolite changes. Regarding the variable effectiveness of *L. suturalis* at some of the New Zealand sites then, it remains uncertain if varying intensity of any phenylpropanoids could be involved. We suggest this question could be addressed with controlled dietary experiments using some of the compounds that were amplified in the NZ range, that are known to impair insect performance.

While plant genetic variability is understood to influence the overall metabolite profile of plants, its effect on metabolite intensity appears equivocal. Literature indicates that for plants of the same species that vary genetically, metabolite intensity is influenced much more by abiotic parameters than genetic variability[88–90]. We acknowledge that genetics may be a contributor to the observed metabolite differences and are currently addressing the degree of genetic variability of *C. vulgaris* between ranges and within sites but posit that differences in soil nutrients and UV-radiation are likely the strongest drivers for these results. Support for both these abiotic influences altering the biochemical profile of *C. vulgaris* in NZ, is provided by recent field trials and a tunnel house experiment using

Site	Olsen P (mg kg^{-1})	Tot N (%/w)	pH	UVI
MU	1.74	0.22 ^{b}	5.94 ^{ab}	
QU	2.17	0.27 ^{ab}	6.06 ^{a}	
WH	1.82	0.19 ^{b}	5.74 ^{b}	
WU	2.63	0.35 ^{a}	5.86 ^{ab}	
$\bar{x}$ **NZ**	**2.09**	**0.26**	**5.9**	**12-13**
BE	9.36 ^{b}	0.57 ^{b}	3.94 ^{b}	
CM	13.2 ^{ab}	1.31 ^{a}	4.31 ^{b}	
GS	25.82 ^{a}	1.64 ^{a}	4.03 ^{b}	
GT	7.41 ^{b}	0.3 ^{b}	4.82 ^{a}	
$\bar{x}$ **SC**	**13.95 ***	**0.96 ***	**4.28 ***	**6-7**

Table 4. Soil nutrient analysis values by site for Olsen P, Total N and pH with all Tukey post hoc levels of significance at $p < 0.05$. Those sites sharing letters are not significant. The New Zealand (NZ) and Scotland (SC) range values differ significantly by paired t-test and are indicated by * with $p < 0.0001$. Summer noontime average UV index for each country is also provided. The NZ Central Plateau UVI values are adjusted for 1000 masl). NZ sites are, Mangatururu (MU), Quarry (QU), Waihohonu (WH) and Waiouru (WU) and for SC are, Ballogie Estate (BE), Creag Meagaidh (CM), Glensaugh (GS) and Glenturret (GT).

UV attenuating screens[58]. From four sites on the CP region of the North Island, significant differences in the volatile organic compound (VOC) emissions of *C. vulgaris* were recorded between sites. Of the environmental variables collected i.e., soil nutrients, ambient daytime temperature, soil water content and soil temperature, the main contributing factor to these differences was soil nutrients[59]. Tunnel house manipulations of ultraviolet light using 20% and 95% attenuating screens and exposing mature and phenologically similar *C. vulgaris* plants for 75 days to this treatment, revealed significant differences between several VOC metabolites, demonstrating that this plant is also sensitive to differing levels of ultraviolet radiation. Which abiotic parameter may be driving the variances revealed in this study however requires controlled experiments manipulating nutrient availability and ultraviolet light both independently and combined, to provide quantitative data to confirm any direct effect of these parameters and further elucidate these results.

Average ambient temperatures vary little between our NZ and SC ranges and soil moisture contents (while we did not measure these) at the time of sampling were within normal summertime ranges, thus we expect little influence on metabolite profiles and intensities from those sources. Induced plant responses to differing insect herbivore communities however needed to be considered. The significantly high number of Hemiptera and Coleoptera at GT was the most likely to produce a positive correlation with high intensities of a given phenylpropanoid at that same site, but none were observed. Additionally, the PCA clusters for GS and CM show considerable overlap with GT, again suggesting that GT is not separating out as a result of any induced response to invertebrate herbivory. There was little evidence then of herbivory influencing the intensity measurements of secondary metabolites which may skew the NZ vs. SC range or inter-site soil nutrient PCA results. Plant ontogeny can influence secondary metabolites which has been reported in a recent paper for VOC compounds in *C. vulgaris*[91], however all plants sampled at all sites in this study were mature and well developed, thus we expect any influence from that source to be negligible. For more in-depth understanding of constitutive metabolites of a successionally dynamic heather community displaying variable plant ontogeny, we also recommend further testing.

While we cannot ascribe direct causality from environmental factors, these results illuminate the potential use of metabolomic techniques for biological control of weeds. Once a potential agent has been selected, metabolomic assays may help determine sites with the closest matching target plant metabolomes, to potentially, both source and release the biocontrol agent. Such information may assist in reducing the chances of encountering difficult establishment or poor effectiveness scenarios. Additionally, it would be of interest to retrospectively compare plant metabolomes from both the source and release sites of already introduced control agents for both successful and unsuccessful programmes to explore to what extent the plant metabolome could be used as a

predictor of biocontrol agent success. Further ideas and potential applications have been extensively discussed in a recent review[37].

Conclusions

Our results clearly demonstrate significant differences between the metabolomes of *C. vulgaris* plants occurring in its native range in Scotland (UK) and those in its invaded range of the Central Plateau of the North Island of New Zealand and suggest that UV-radiation and soil nutrients could be driving the observed differences. However, to assess direct causality further experiments under controlled conditions are necessary.

It is also evident for sites within each of the ranges we tested, significant variation occurs for many phenylpropanoids. Bioassays exploring biocontrol agent behaviour, feeding preferences and life history performance are required to understand if both inter and intra site variance in this and potentially other classes of compounds could be a driver of poor agent establishment rates or variable effectiveness.

Our non-targeted metabolomics approach to these investigations revealed not just secondary defensive metabolites, but the majority of the primary amino acid metabolites identified (in addition to those key precursors of the shikimate-phenylpropanoid pathway i.e., phenylalanine and tyrosine) are also amplified suggesting overall increased biosynthetic pathway upregulation of this plant in the New Zealand CP environment. Our results therefore add to the very limited literature documenting biochemical phenotypic change to invasive plants that have established in a new, abiotically different region.

We conclude, this study has provided a primer, that abiotically induced biochemical change may not be uncommon in invasive plants. This validates our original question of the potential for biochemically modified phenotypes altering plant defensive capacity and/or nutritional assimilability which may compromise specialist control agents when reunited with their coevolutional host plant. The application of metabolomics therefore, may be a valuable tool to assist with determining such changes and help elucidate poor establishment and/or effectiveness scenarios in weed biocontrol.

Data availability

All relevant .raw spectral data sets are available upon reasonable request.

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Author contributions

DPB. Conceived the primary questions and objectives of the investigations. Carried out all sampling, processing of samples, annotation and statistical analysis. Secured funding. Primary author and writer of the manuscript. AKS. Advised on appropriate chromatographic analysis, supervised and ran all UHPLC-MS technical processes, advised regarding metabolomic analysis techniques and revised the manuscript.RJP. Advised and assisted with site selection and sample collection carried out in Scotland UK. Facilitated hosting and access to laboratory facilities at the James Hutton Institute, Aberdeen and revised the manuscript.PP. Has provided 2 decades worth of background research information on the Heather biocontrol program. Now recently has provided expertise on foliar N impacts on heather beetle control agent performance and other invertebrates associated with this program and revised the manuscript.ACM. Principal investigator and project supervisor. Advised on concept, design and interpretation of investigations, supported fieldwork sampling, secured funding and revised the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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